Supplementary Information

Antiviral CD8⁺ T cell immune responses are impaired by cigarette smoke and in COPD

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I. Supplementary Figures

Figure S1: Immunoproteasome gene expression upon virus infection and IFNγ stimulation

a) scRNA expression of immunoproteasome subunits from influenza-infected and mock-infected lungs derived from the publicly available data set GSE107947 [1]. Clustered cells were annotated as unexposed, infected or bystander cells. **b)** RNA expression of the immunoproteasome subunits *Psmb8*, *Psmb9* and *Psmb10* from alveolar epithelial cells type II (AECII) isolated from influenza-infected lung tissue (n=2) [2]. **c)** MTT assay after 24h of treatment with increasing doses of CSE, mean ± SEM of four independent experiments (n=4). Metabolic activity was normalized to untreated controls (100%). **d)** *Psmb8*, *Psmb9* and *Psmb10* mRNA expression in MLE-12 cells co-treated with or without 10% or 25% CSE and/or 75 IU/ml IFNγ for 24 hours. Expression levels of co-treated cells were normalized to *Rpl19* and *Hprt* and then normalized to IFNγ-treated cells in four independent experiments (n=4). **e)** LMP2 and LMP7 expression in mouse precision cut lung slices (PCLS) treated with 10% or 25% CSE and 75 IU/ml IFNγ for 24 hours. Densitometric analysis of LMP2 and LMP7 expression was normalized to β-actin with the IFNγ-only control set to 1. Statistics (d and e): one sample *t* test with $*p<0.05$, $**p<0.01$.

Figure S2: Cigarette smoke attenuates induction of immunoproteasome by PolyI:C or MHV-68 infection

a) Relative mRNA expression of *Psmb8*, *Psmb9* and *Psmb10* in murine lungs 2, 8, and 24 hours after a single treatment with 10 µg PolyI:C in mice that had been cigarette smoke exposed for 24 days. Expression values were normalized to *Hprt* and *Tbp* housekeeping genes using the $2^{-deltact}$ method and then normalized to relative mRNA levels of air-treated animals. Significance was calculated by Two-way ANOVA (Post hoc test: Tukey) **b)** Paraffin sections of mice (3 mice/group) exposed to air or cigarette smoke for 24 days and then treated with PBS or 10 µg PolyI:C for 24h were stained for LMP2. Representative images also of indicated control stainings are shown. **c)** Weight curve of mice that had been exposed to cigarette smoke for 28 days up to seven days after MHV-68 infection (5×10^4 PFU). Two-Way ANOVA with Bonferroni's post test (compared to air mock) $***p<0.001$. **d)** qPCR analysis of viral immediate-early transactivator ORF50 ratio to host GAPDH DNA levels. Mann-Whitney-U test. **e)** Flow cytometry analysis of lung $CD3^+$ T cells and **f)** $CD8^+$ T cells as percentage of all gated living cells. Kruskal-Wallis-Test with Dunn's post test. n.s. not significant.

Figure S3: Cigarette smoke attenuates IAV-induced upregulation of the immunoproteasome in human epithelial cells.

a) pBAECs were isolated from a single donor were exposed to CSE for 43 days at air-liquid-interface conditions and then infected with Influenza A Virus (IAV, MOI 1) for 1 or 3 days to determine the kinetic of immunoproteasome induction. LMP2 and LMP7 was analyzed by Western blotting with β-actin serving as a loading control. MLE12 cells treated with IFNγ served as a positive control. **b)** Expression of LMP2 and LMP7 in A549 treated with 20% CSE and infected with Influenza A virus (IAV, MOI 1) for 24 or 48 hours. Densitometric analysis of LMP2 and LMP7 expression normalized to β-actin loading with controls set to 1 (mean ± SEM). Statistical analysis One-Way ANOVA with Bonferroni post test. $^{**}p<0.01$. **c)** MTT assay of A549 cells treated for 24 hours with increasing doses of CSE, Metabolic activity was normalized to untreated controls (100%). Depicted is the mean ± SEM of three independent experiments (n=3).

Figure S4: Effects of CSE on IAV-infected primary human lung fibroblasts (phLF).

a) MTT assay of phLFs following exposure to increasing doses of CSE for 24h in three independent experiments (n=3). Metabolic activity was normalized to untreated controls (100%). **b**) Immunoproteasome subunits LMP2 and LMP7 expression in phLF treated with 20% CSE and infected with IAV (MOI 1). Densitometric analysis of LMP2 and LMP7 expression normalized to β-actin with control set to 1. **c**) IAV-specific $CD8^+$ T cell activation upon co-culture of T cells with phLF that had been treated with IFNγ for 24 hours and incubated with the $M1_{58-66}$ peptide GILGFVFTL. T cell only and T cell co-cultured with phLF pre-treated with CSE serve as negative controls. Data are shown as the mean±SEM from three independent experiments. Significance was tested with Student's t test test with $^{***}p<0.001$.

Figure S5: CSE treatment of IFNγ-treated A549 cells.

a) LMP2 and LMP7 expression in A549 cells exposed to 20% CSE every day for 72 h and treated with or without 75 IU/ml IFNγ for the last 24 h. Densitometric analysis of LMP2 and LMP7 expression normalized to β-actin with control (Ctrl) set to 1 (three independent experiments). One-Way ANOVA with Bonferroni post test, n.s. not significant **b)** HLA-ABC surface expression on A549 cells treated with 20% CSE and 75 IU/ml IFNγ for 24 h. Analysis of mean fluorescence intensity (MFI) shows mean ± SEM of three independent experiments (n=3).

Figure S6: Immunopeptidomic analysis in A549 cells.

a) Stacked bar plot of peptides frequently identified in immunopeptidome preparations of treated A549 cells. The 8-14mer peptides were grouped into HLA specific peptides (“Binder”; binding rank ≤ 2%) and unspecific peptides (“Non-Binder”). **b)** Depiction of the relative contribution of the HLA alleles to the immunopeptidome. HLA-specificity is shown as mean± SD of identified peptides upon treatment with CSE, IFNγ or CSE/IFNγ. The HLA-specific peptides (binding rank ≤ 2%) were grouped by the HLA allele that showed the smallest HLA binding rank, as calculated using the MixMHCpred software tool (version 2.1). A non-parametric Aligned Rank Transform (ART) ANOVA, followed by ART Contrasts post hoc test was performed to calculate p-values. The p-values were corrected for multiple testing with the method of Benjamini and Hochberg, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. **c)** Abundance comparison of peptides identified in the immunopeptidome and proteins detected in the proteome from CSE, IFNγ or CSE/IFNγ-treated cells, each compared to control cells alone. Significantly upregulated MHC class I peptides are depicted in red, while significantly downregulated MHC class I peptides are in blue. Proteins significantly regulated in the proteome without significant changes in the peptidome are depicted in black. Peptides significantly regulated in the proteome and the immunopeptidome are labeled with their gene symbol. Enumeration of peptides is given in the respective tables. Thereby, (-) stands for significant downregulation and (+) stands for significant upregulation.

Figure S7: Gating strategy for IAV-tetramer$^+$ CD8$^+$ T cells.

a) To identify the IAV-specific CD8$^+$ T cells and the T cell subpopulations, the lymphocyte population was determined and distinguished from monocytes by FSC and SSC (1). From the lymphocyte population duplicates were excluded (2) and live cells were distinguished from dead cells (3). Within the living single lymphocyte population, all CD3$^+$ T cells were detected (4) to subsequently determine the proportion of IAV-specific CD8$^+$ T cells in all CD3$^+$ T cells (5) and in all CD8$^+$ T cells (6). IAV-specific T cells were gated as specific population only if they were visible as a population clearly distinct and not merely an extension of the main CD8 stained population. **b)** HLA-A2 positive subjects without tetramer staining as well as **c)** HLA-A2 negative subjects were used to determine the specificity of the tetramer staining.

Figure S8: Determination of Influenza A virus specific CD8$^+$ T cells in blood of healthy controls and asthma patients.

a) Absolute numbers of CD3$^+$CD8$^+$IAV-Tetramer$^+$ T cells (cells/μl) in the lung healthy controls (n=10) and asthma patients (n=12) **b)** Percentage of CD8$^+$IAV-Tet$^+$ T cells within the fraction of all CD3$^+$ cells in PBMCs of lung healthy controls (n=8), or asthma patients (n=12). Significance was tested with Mann-Whitney U test with $*p < 0.05$.

II. Materials and Methods

Human samples

EDTA-Blood samples of 102 donors (healthy, COPD or asthmatics) were obtained of which 35 HLA-A2 positive donors were used for tetramer analysis from LMU Hospital and the outpatient unit of the Comprehensive Pneumology Center as part of the CPC-M bioArchive at the Comprehensive Pneumology Center (CPC Munich, Germany). Age-matched lung-healthy control subjects were allocated from the Helmholtz Center Munich (Table 1 for patients' characteristics). All enrolled donors gave written consent, and the study was approved by the ethics committee of the LMU medical faculty under the study number 382-10. For the never-smoker/ever smoker cohort, DNA from 81 healthy volunteers available from the BioMaterialBank Nord was screened by PCR for HLA-A2 status [3]. From the 29 HLA-A2-positive donors, we isolated PBMCs from 7 never-smokers (including one Shisha smoker) and 7 ex- or current-smokers. The study was approved by the local ethics committee of the Universität zu Lübeck, Germany (Ethic votes 14-225 and 22-583).

Supplementary Table S1: Characteristics of never and ever-smokers

	% or median(range)	n/N or N
sex		
female	50	7/14
male	50	7/14
age,years	35(51;21)	14
BMI[A],kg/m²	24.25(18.71;37.04)	14
comorbidities[B]		
no	100.00	14/14
yes	0.00	0/14
smoking status		
current	50	7/14
never	50	7/14
pack years	14.5(0.2;22)	7/14

[A] Body Mass Index; [B] defined as hypertension, myocardial infarction or stroke.

Cell lines, primary cells, and treatments

The following cell lines were used with medium supplemented with 10% fetal bovine serum (Biochrom) and 1x Pen/Strep (Gibco) and were cultured in a humidified atmosphere with 5% CO_2 at 37°C: MLE-12 (mouse lung epithelial-like cell line, RPMI-1640 medium (Gibco)), A549 (human lung epithelial-like adenocarcinoma cell line, DMEM). Mouse precision-cut lung slices (PCLS) were prepared from C57BL/6 mice with a thickness of 300 µm as previously described [4, 5] and cultured in (DMEM-F12 medium containing 10% fetal bovine serum (Biochrome),

100 U/ml Penicillin/Streptomycin (Gibco) and 2.5 µg/mL amphotericin B (Gibco). Primary human lung fibroblasts from two individual donors (HLA-A2$^+$) were used at passages <8 as described [6]. Primary human bronchial epithelial cells (phBECs) were cultured ± CSE and infected with IAV as described [7]. Human peripheral blood mononuclear cells were isolated from 16 ml blood using SepMate^{TM} and Lymphoprep^{TM} (Stemcell Technologies).

Cigarette smoke extract for cell culture experiments was prepared by bubbling the smoke of 6 cigarettes (3R4F, Kentucky University) through 100 ml cell culture medium without supplements. Afterwards, medium was sterile-filtered and stored at -20°C. Mouse recombinant IFNγ (Roche, 11276905001) or human recombinant IFNγ (Roche, 11040596001) was used at 75 IU/ml, PolyI:C (Sigma) was titrated for highest induction of the immunoproteasome without cytotoxicity and applied with 1 or 5 µg/ml via electroporation of cells with 500 V and 20 ms. PCLS were cultured with 1 µg/ml PolyI:C.

Virus stock production

H1N1 influenza virus A/Puerto Rico/8/34 (PR8) and Madin-Darby canine kidney (MDCK) cells were kindly provided by Susanne Herold (University of Giessen and Marburg Lung Center). MDCK cells were cultured in minimum essential medium (MEM) (Gibco) supplemented with 10% FCS (PAN Biotech) and penicillin/streptomycin (100 U/ml) (Gibco). Cells were infected with PR8 as described below, and the supernatant was collected when complete cytopathic effect (cpe) was reached. Aliquots of the supernatant were stored at -80°C. The virus titer was determined using MDCK cells. Briefly, 10-fold dilutions of the virus stock were prepared in MEM containing 0.1% BSA, penicillin/streptomycin and trypsin (5 µg/mL) (Sigma) and, after washing the cells once with serum-free MEM, 100 µl of each dilution were added in quadruplicate to the wells of a 96-well plate. The cpe was examined by phase contrast microscopy 72 hours after infection. The dilution at which 50% of the wells showed cpe was used to calculate the median tissue culture infectious dose ($TCID_{50}$) by the method of Reed and Muench [8]. Murine gammaherpesvirus 68 (MHV-68) was grown and titrated on BHK-21 cells as previously described [9]. Purified HRV-16 virus was purchased from ATCC (VR-283PQ, LOT:64541367).

Treatment of NHBE cells with CS and HRV-16 at air-liquid interface

Primary normal human bronchial epithelial (NHBE) cells from two healthy male donors, aged 52 and 54 (Lonza), were cultured in T25cm^2 flasks (Sarstedt) in bronchial epithelial cell growth medium (BEGM) (Lonza). When confluency reached 70%, cells were passaged and seeded at a density of 9×10^4 cells/cm^2 on 12-well transwell inserts (transparent, 0.4 µm. Corning) coated with human placental collagen type IV (Sigma-Aldrich) in BEGM media. To do air lift (d0), the apical medium was aspirated, and the basal medium was substituted with

PneumaCult-ALI media (Stemcell Technologies) containing 1% penicillin/streptomycin (PAN biotech) after approximately two days of expansion. For differentiation into a pseudo-stratified epithelium, the NHBE cells were left at air-liquid interface with regular changes of the basal medium every two days. The apical mucus was removed by weekly washes with 500 µl of Hanks' Balanced Salt Solution (HBSS). NHBE cells were exposed to cigarettes smoke (CS) (3R4F research cigarettes, Univ. of Kentucky, USA) every day from day 14 to day 27 post air-lift using the P.R.I.T.® ExpoCube® system (Fraunhofer ITEM, Hannover, Germany), connected to a cigarette smoking robot (In-Expose system, Scireq, Canada) as described [10]. CS was generated using 2 cigarettes per day (1 puff/min), and the exposure was controlled by flexiWare software Version 6.1 and monitored for particle mass and CO via photometer and CO-monitors, respectively, with the DASYLab software (Version 13, National Instruments, Munich, Germany). Subsequently, cells were infected with human rhinovirus 16 (HRV-16) (ATCC, USA) at day 28 post air-lift, by exposing apical part of cells to an infection with HRV-16 at a multiplicity of infection (MOI) of 1 for 2 hours. Cells were sampled for RNA expression analysis at day 30.

Supplementary Table S2: Summary of NHBE donor information (Lonza)

Lot No.	Weight	Height	Age	Sex	Race	Smoking
20TL266556	80 kg	172 cm	52 years	male	Hispanic	No
20TL356518	76 kg	168 cm	54 years	male	Caucasian	No

Infection of cells and PCLS

Cultured cells were washed twice with prewarmed HBSS. Subsequently, cells were infected with PR8 diluted in HBSS at a MOI (multiplicity of infection) of 1. Plates were gently rocked several times and incubated at 37°C for 1 hour. After removing the inoculum, cells were washed twice with HBSS, and the previous medium was added again for further culturing. The cells were incubated at 37°C and 5% CO_2 until analysis.

Mouse precision cut lung slices (PCLS) were infected with $5x10^4$ PFU of MHV-68 per slice, leaving the inoculum on the PCLS for the whole culture period until analysis.

Human lung epithelial cells A549 were seeded in 6-well plates. A549 cells were pre-exposed to 20%CSE for 24 h. Subsequently, cells were infected with H1N1 influenza virus A/Puerto Rico/8/34 (IAV) diluted in HBSS at a MOI of 1 for 1 h. After infection, cells were washed twice with HBSS, and the previous 20%CSE medium was added again for further 24 to 48 hours of culturing.

Mouse cigarette smoke and PolyI:C exposure

All animal experiments were approved by the Ministry of Energy, Agriculture, the Environment, Nature and Digitalization, Schleswig-Holstein, Germany (V 242 – 41093/2016 [82-7/16]). Mice were exposed to cigarette smoke generated from 3R4F Research Cigarettes (University of Kentucky, USA) by a smoking robot as described earlier [10]. In brief, mainstream cigarette smoke (CS) as a surrogate for active smoking was drawn into a whole-body exposure chamber connected to the smoking robot (in Expose System, Scireq; flexiWare Version 6.1). In this manner, animals were exposed to CS for 1 hour daily for 24 days (smoking 6 cigarettes (1 puff/min) each day for 3 days and then 24 cigarettes (4 puffs/min) for 21 days. Control animals were exposed to air. Serum cotinine (Calbiotech, El Cajon, USA) and lung *Cyp1a1* expression were assessed routinely to ascertain the exposure. Stable running of the robot was monitored by on-line particle measuring (Casella, Bedford, UK) at the smoke outlet of the exposure chamber [10]. 10 µg PolyI:C (polyinosine polycytidylic acid; Sigma-Aldrich, St. Louis, USA) was dissolved in 40 µl phosphate-buffered saline (PBS) and administered intranasally under mild inhalation anesthesia (sevoflurane) 1 h after the last CS exposure (day 24). PBS was used as a control application. Mice were sacrificed after 2, 8 or 24 h and lungs were collected and snap frozen until analysis. For extraction of proteins or RNA, lungs were placed in a mortar with liquid nitrogen and crushed with a pestle. The lung powder was aliquoted at 30 mg weight into 2 ml reaction tubes. For purification of total RNA from the lung tissue samples, the lung powder was dissolved in Qiazol® (Qiagen, Hilden, Germany) and isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany).

For immunohistochemistry, lungs were embedded in paraffin after bronchoalveolar lavage procedure and processed according to standard procedures. Antigen retrieval was achieved by cooking for 10 min in a pressure cooker in citrate buffer (0.01M, pH 6) Upon cooling of the slides for 15 min, slides were rinsed with H_2O_2dest for 5 min and washed twice for 5 min each in TBS buffer (0.01M, pH 7.4). For staining, the LMP2 antibody (ab242061, Abcam) was applied at 1:2000 dilution over night at 4°C using cover plates (Epredia). Upon washing in TBS buffer for 5 min, slides were washed in TBST (0.05M TBS, pH7.4 +0.05% Tween20) twice for 2 min each and incubated with anti-Rabbit-AP Polymer (#05269709001, Roche) diluted 1:4 in antibody dilutent solution (ZUC025-500, ZytoMed) for 10 min at RT. Slides were again washed twice in TBST for 2 min and substrate reaction was performed using the Universal Alkaline Phosphatase Red Detection Kit (Roche) according to manufacturer's recommendations. Slides were then rinsed in H_2O_2dest, counterstained with hematoxyclin (H3136, Sigma Aldrich) for 2 min, drained and embedded into entellan (#1.07961, Merck). Pictures were taken using the Nikon Eclipse 80I microscope and the photos were processed using the software "Fix Foto" (Joachim Koopmann).

Mouse cigarette smoke exposure and infection with MHV-68

All animal protocols were approved by the IACUC of the University of Pittsburgh and adhered to NIH guidelines for the use of experimental animals. Female C57BL/6J (The Jackson Laboratory #000664), at 10 weeks of age, were exposed to short-term cigarette smoke (CS) exposure using a total body cigarette smoke exposure system comprised of a stainless-steel chamber and a smoking machine that delivered a combination of side-stream and main-stream smoke (model TE-10; Teague Enterprises, Woodland, CA). The method of environmental tobacco smoke exposure has been previously reported [11]. The cigarette smoke from 100 cigarettes (4R3F University of Kentucky research cigarettes) was delivered to the mice each day, 5 days per week for approximately 2.5 h in duration each day. Control mice were exposed to filtered air only under the same environmental conditions.

At day 28, mice were infected with 5 x 10^4 PFU. MHV-68 dissolved in 40 µl pf DMEM (Gibco) intranasally as previously reported [12]. Control animal received 40 µl of the same DMEM without virus. Group size was n=8. Weight loss was monitored daily. Seven days post infection, animals were sacrificed, and the right lung lobes were snap-frozen until analysis. In the group of air + MHV-68, one animal had to be excluded due to very low viral DNA copy numbers and without weight loss, two animals were excluded in the CS + MHV-68 group for the same reasons.

Flow cytometry of mouse lungs: The left lung lobe was used for flow cytometry analysis by cutting it into small pieces and digestion in PBS with collagenase D (2 mg/ml) and DNase (10 µg/ml) at 37°C for 45 min. Cells were pushed through 40 µm mesh using a piston and collected in FACS buffer (PBS, 0.1% BSA, 0.1% NaN_3), centrifuged and resuspended in FACS buffer. Two million cells were stained with Zombie Aqua fixable viability kit, CD3-Pacific Blue or APC-CD8 antibodies or appropriate isotype controls. Cells were fixed with PFA, washed and kept on ice until analysis.

DNA isolation and MHV-68 copy numbers: Purification of DNA was performed in spleen samples using DNeasy kit (QIAGEN) and from lung using AllPrep DNA/RNA Minikit (Qiagen) according to the manufacturer's instructions. Quantitative PCR to determine viral copies of MHV-68 DNA was performed in spleen samples as described previously [12], using viral ORF50 primers (ORF50 fwd: GGCCGCAGACATTTAATGAC, ORF50 rev: GCCTCAACTTCTCTGGATATGCC) and host GAPDH as housekeeping gene (GAPDH fwd: CCTGCACCACCAACTGCTTAG, GAPDH rev: GTGGATGCAGGGATGATGTTC). Cycling conditions were as follows: 95°C for 10 min and then 40 cycles at 95°C for 15 s, 60°C for 1 min (Applied Biosystems 7300 Real Time PCR), using VeriQuest SYBR Green qPCR Master Mix (Affymetrix). The number of copies of viral ORF50 and cellular GAPDH in each sample was determined by comparison to a series of standard curve reactions using a plasmid control containing the ORF50 and GAPDH sequences (melting curve analysis was performed to verify

specificity). The standard curve dilutions used represented a range from 10^8 to 10^2 copies, in serial 10-fold dilutions of a cloning plasmid containing cDNA of ORF50 and murine GAPDH.

Antigen presentation assay

Influenza M1-specific 4VA1 T cells were kindly provided by David Canaday (Case Western Reserve University, Cleveland, Ohio, USA) [13], cultured in DMEM High glucose medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 4 mM L-glutamine (Thermo Fisher Scientific), 100 U/ml penicillin/streptomycin (Thermo Fisher Scientific), 1% HEPES (Sigma-Aldrich), 1% non-essential amino acid (Euroclone) and 25 µM 2-mercaptoethanol (Thermo Fisher Scientific).

phLF were plated in 12-well culture plates and treated with 10% CSE in 1% FBS for 24 h. Afterwards cells were infected with IAV with MOI of 1 for 1 h and further cultured for 24 h +/- CSE. Positive control cells were treated with 75 IU/ml IFNγ for 24 h. 1 µg/ml Flu-M1 peptide (Genscript, RP19978) was added into the medium for 90 min and cells then washed with PBS. A separate well was used for counting the cells using trypan blue exclusion assay (Thermo Fisher Scientific, 15250061) in order to determine the exact cell number for T cell co-culturing. phLF cells were co-cultured with the $CD8^+$ Influenza-M1 protein specific T cells (4VA1 $CD8^+$ T cells) at the ratio of 1:2 for 24 h. The supernatant was then harvested and centrifuged at 1,500 rpm for 5 min. IL-2 secreted by the mouse-derived T cell clone was detected in the supernatant using the mouse IL-2 MAX™ standard set (Biolegend, 431001) and DuoSet® Ancillary Reagent Kit 2 (R&D systems, #DY008). Secretion of IL-2 per $CD8^+$ T cells was calculated by multiplying the measured concentration of IL-2 with the volume of supernatant of 4VA1 $CD8^+$ T cell and phLF co-cultures in ml divided by $CD8^+$ T cell numbers.

Flow cytometry of human blood cells

EDTA-blood of COPD patients (n=31), Asthma patients (n=47), or healthy controls (n=31) was stained with antibodies targeting lineage markers to analyze the number of subpopulations of granulocytes (neutrophils and eosinophils), monocytes (classical, non-classical or intermediate monocytes), and lymphocytes (T cells, B cells and natural killer cells). For details on the antibodies used see Supplementary Table S2. An overview of the markers used and the gating strategy can be seen in Supplementary Table S3. Briefly, 100 µl of blood was mixed with the antibodies mentioned in Supplemental Table S2. After 20 min incubation on ice in the dark, erythrocyte lysis was performed using the TQ-Prep workstation according to the manufacturer's instructions (Beckman Coulter). 100 µl of count fluorospheres (Beckman Coulter) of known concentration were added prior to the start of the measurements of the cell populations on an LSR II flow cytometer (Becton Dickinson) operated with Diva software Version 8.0. For determination of $CD4^+$ and $CD8^+$ T cells, PBMCs were isolated using the

SepMate tubes and Lymphoprep solution (StemCell) according to the manufacturer's instructions and then frozen in RPMI Medium (Gibco) containing 1x Pen/Strep, 10% fetal bovine serum (FBS Superior, Biochrom) and 5% DMSO. Analysis was performed in the same setup when IAV-tetramer staining was performed (see below). Compensations were performed using compensation beads (Beckman Coulter) and specific staining was confirmed by using Fluorescence-minus-one (FMO) control staining.

Supplementary Table S3: Antibodies for whole blood analysis

antibody	fluorochrome	clone	isotype	dilution	company
anti-CD15	FITC	W6D3	IgG1, κ	1:20	Becton Dickinson, Franklin Lakes, USA
anti-CD16	PE	3G8	IgG1	1:100	Beckman Coulter, Brea, USA
anti-CD14	BV421	M5E2	IgG2a, κ	1:100	Becton Dickinson, Franklin Lakes, USA
anti-CD3	PerCP-Cy5.5	SK7	IgG1, κ	1:20	Thermo Fisher Scientific, Waltham, USA
anti-CD56	APC	N901	IgG1	1:10	Beckman Coulter, Brea, USA
anti-CD19	APC-Cy7	SJ25C1	BALB/c IgG1, κ	1:50	Becton Dickinson, Franklin Lakes, USA

Supplementary Table S4: Summary of gating strategy

cell population	gating strategy
leukocytes	FSC, SSC
lymphocytes	FSC, SSC
T cells	starting from the leukocytes: inclusion of CD3+ cells, then exclusion of all $CD14^+$ cells and $CD16^+$ cells.
B cells	starting from the leukocytes: inclusion of $CD19^+$ cells, then exclusion of all $CD14^+$ cells, $CD56^+$ cells and $CD16^+$ cells.
natural killer cells	starting from the leukocytes: exclusion of $CD3^+$ cells, $CD19^+$ cells and $CD14^+$ cells, then inclusion of $CD56^+$ cells.
monoyctes	FSC, SSC
monocyte subpopulations	starting from the monocytes: exclusion of $CD19^+$ cells and $CD56^+$ cells, then determination of monocyte subpopulations based on $CD14^+$ and $CD16^+$.
granulocytes	FSC, SSC

granulocyte subpopulations	starting from the granulocytes: exclusion of $CD14^+$ cells, $CD56^+$ cells and $CD3^+$ cells, then determination of neutrophils as $CD15^+$ and $CD16^+$ and determination of eosinophils as $CD15^+$ and $CD16^-$

HLA-A2 status determination

HLA-A2 determination was performed by using flow cytometry using HLA-A2-FITC (clone BB7.2) compared to an isotype control (clone MG2b-57) (Biolegend) in whole blood using the same protocol as mentioned above. Furthermore, results were confirmed by isolating genomic DNA with the DNeasy blood and tissue kits (69504, Qiagen) and performing genotyping using 25 ng DNA and 100 pM HLA-A2 specific primers (FW 5'-CCTCGTCCCCAGGCTCT-3', REV 5'-GTCCCAATTGTCTCCCCTC-3') on a Biorad PCR machine with 40 cycles (95°C 30'', 60°C 1 min, 72°C 1 min).

IAV tetramer staining

Freshly thawed PBMCs were stained with antibodies targeting CD3, CD8a, CD4 and the APC-HLA-A2 tetramer loaded with the IAV immunodominant $M1_{58-66}$ peptide GILGFVFTL (Supplemental Table S3). These tetramers were obtained by the NIH Tetramer Core Facility (contract number 75N93020D00005). Briefly, cells were carefully thawed and $1x10^6$ living cells were incubated with the tetramer in 100 µl FACS buffer (PBS containing 2 mM EDTA and 2% FBS Superior) for 30 min at room temperature in the dark. For each sample, a control containing no tetramer was analyzed in parallel. Afterwards, cells were washed and incubated with antibodies as well as Live/Dead Fixable Blue Dead Cell dye (Thermo Fisher Scientific) for 20 min on ice in the dark. For the never-/ever-smoker cohort, Live/Dead staining was done for 20 min at RT before antibodies were added. After one more washing step, analysis was performed. HLA-A2 negative samples and samples without tetramer staining were used as controls, the full gating strategy is displayed in Supplemental Figure S6. For the second study arm, we added an additional gating step (4b) where we discriminated for $CD3^+$ cell aggregates by plotting CD3 against SSC-width. Unspecific background was excluded by staining for the cells in the absence of tetramers. Absolute $Tetramer^+$ cells/µl blood were calculated by using the percentage of $CD8^+Tetramer^+$ T cells of $CD3^+$ T cells in the PBMCs and the absolute amount of $CD3^+$ T cells per µl blood from the whole blood analysis as described above, where counting beads were used. For the never/ever smoking arm we did not use any counting beads and only display the percentage of $CD3^+CD8^+$IAV-Tet^+cells as percentage of all $CD8^+$ T cells.

Supplementary Table S5: Antibodies for PBMC tetramer analysis

antibody/tetramer	fluorochrome	clone/peptide	isotype	dilution	company

anti-CD3	Per-CP-Cy5.5	SK7	IgG1, κ	1:20	Thermo Fisher Scientific, Waltham, USA
anti-CD8a	FITC	RPA-T8	IgG1, κ	1:20	BioLegend, San Diego, USA
anti-CD4	APC-H7	RPA-T4	IgG1, κ	1:20	Becton Dickinson, Franklin Lakes, USA
IAV-tetramer	APC	GILGFVFTL	-	1:100	NIH Tetramer Core Facility, Atlanta, USA

Flow cytometry determination of HLA regulation by CSE

A549 were plated in 12-well culture plates and treated with 20% CSE in 1% FBS for 24 h or left untreated in medium containing 1% FCS. Cells were then infected with IAV diluted in HBSS at a MOI of 1 for further 24 h. Afterwards, cells were trypsinized and fixed with 4% PFA for 5 min. Antibody HLA-ABC Alexa488 (W6/32, Biolegend) was employed for staining in a 1:100 dilution for 30 min at 4°C. After further washing of the samples with FACS buffer, fluorescent signals were detected by flow cytometry analysis using the Becton Dickinson BD™ LSR II Flow Cytometer System (BD Biosciences-Europe), for both study arms with the same configuration and the FlowJo software (TreeStart Inc; Ashland, USA, version 7.6.5) as well as the FCS express software (De Novo Software; Pasadena, USA, version 7.12.0020).

MHC class I immunoprecipitation and peptide elution

A549 cells were grown in 15 cm dishes, 10 dishes per replicate were used for control and IFNγ treatment, while 20 dishes per replicate were used for CSE treatment. Cells were treated with fresh 20% CSE-containing medium for 3 days. On the third day, cells were treated with 75 IU/ml IFNγ for 24 h either in the presence or absence of CSE. Cells were then scraped in PBS, counted, centrifuged and pellets were stored at -80°C for analysis. 100 million cells were lysed with a buffer containing 0.25% (w/v) sodium deoxycholate, 0.2 mM iodoacetamide, 1 mM EDTA, 1x cOmplete™ Protease Inhibitor Cocktail (Roche), 1 mM Phenylmethyl-sulfonylfluoride, 1% Octyl-beta-D-glucopyranoside in PBS [14]. 15 µl of each lysate were transferred into freshly prepared 1.5 mL Lo-Bind tubes (Eppendorf AG) for subsequent proteome analysis. Protein concentration of the lysates for proteome analysis were determined by Pierce BCA assay (Thermo Fisher Scientific). 10 µg per lysate were digested with Lys-C and trypsin using a modified FASP procedure as described elsewhere [15, 16].

Lysates for immunopeptidomics analysis were cleared by centrifugation (51 200 g for 50 min at 4°C) and native MHC class I complexes were precipitated and purified with the pan-MHC class I specific antibody W6/32 (HB-95 ATCC) coupled to protein G sepharose 4 Fast Flow beads (GE Healthcare) as described [14]. Co-precipitated immunopeptides were resolved with 1% (v/v) trifluoroacetic acid (TFA) and purified using Sep-Pak tC18 columns containing 100 mg sorbent (Waters). Elution of peptides from the tC18 sorbent was conducted in two steps, first with 28% acetonitrile (ACN) in 0.1% TFA and consecutively with 32% ACN in 0.1% TFA. Both eluates were volume reduced using a vacuum evaporator until almost all liquid was evaporated. Peptides were then resolved with 2% ACN in 0.5% TFA and stored at -80 °C until further analysis [14].

LC-MS/MS and Quantitative Analysis

LC-MS/MS analysis was performed on a QExactive HF-X mass spectrometer (Thermo Fisher Scientific) online coupled to a Ultimate 3000 RSLC nano-HPLC (Dionex, Sunnyvale, United States). For total proteome analyses, peptides from FASP digestion were automatically injected and loaded onto a C18 trap column (300 µm inner diameter (ID) × 5 mm, Acclaim PepMap100 C18, 5 µm, 100 Å, LC Packings) at 30 µl/min flow rate prior to C18 reversed phase chromatography on the analytical column (nanoEase MZ HSS T3 Column, 100Å, 1.8 µm, 75 µm x 250 mm, Waters) at 250 nl/min flow rate in a 95 minutes non-linear acetonitrile gradient from 3 to 40% in 0.1% formic acid. Profile precursor spectra from 300 to 1500 m/z were recorded at 60000 resolution with an automatic gain control (AGC) target of 3e6 and a maximum injection time of 30 ms. The 15 most abundant peptide ions of charges 2 to 7 were selected from the MS scan and fragmented by HCD with anormalized collision energy of 28 and isolation window of 1.6 m/z, and a dynamic exclusion of 30 s. MS/MS spectra were recorded at a resolution of 15,000 with an AGC target of 1e5 and a maximum injection time of 50 ms. This method was adapted for immunopeptidome measurements as follows: recording the profile precursor spectrum from 300 to 1650 m/z at 60 000 resolution, top 15 fragment spectra of charges 1 to 5 were recorded at 30 000 resolution and a dynamic exclusion for 15 seconds.

The Proteome Discoverer 2.5 software (version 2.5.0.400; Thermo Fisher Scientific) was used for peptide and protein identification via a database search (Sequest HT search engine) against the SwissProt Human database (Release 2020_02, 20435 sequences; 11490581 residues). Furthermore, the workflow for the identification of the immunopeptidome included the INFERYS rescoring node [17]. Database search was performed with full tryptic specificity and allowing for up to one missed tryptic cleavage site (proteome) or with unspecified peptide cleavage (immunopeptidome), respectively. The precursor mass tolerance was 10 ppm and the fragment mass tolerance was 0.02 Da. Carbamidomethylation of Cys was set as static

modification. Dynamic modifications included deamidation of Asn and Gln, oxidation of Met, and a combination of Met loss with acetylation on protein N-terminus. Peptide spectrum matches and peptides were validated with the Percolator algorithm [18].

The proteomes and corresponding immunopeptidomes of three independent experiments were analyzed, with the latter measured in technical duplicates. Only the top-scoring hits for each spectrum were accepted with a false discovery rate (FDR) < 1% (high confidence). The final list of proteins from the total proteome satisfied the strict parsimony principle and included only protein groups passing an additional protein confidence filter FDR <5% filter (target/decoy concatenated search validation). Quantification of proteins, after precursor recalibration, was based on intensity values (at RT apex) for all unique peptides per protein. Peptide abundances derived from proteome samples were normalized to the total peptide amount. The protein abundances were calculated summing the normalized abundance values of corresponding unique peptides.

The immunopeptides were not normalized, to prevent distorted protein abundances due to differences in protein HLA class I presentation. Protein abundances were used for calculation of enrichment ratios of proteins in the stimulated samples to the untreated samples, resulting in single ratios for every quantified protein in every treated sample. Significance of the ratios was tested using a background-based t-test with correction for multiple testing according to Benjamini-Hochberg (adjusted p-value) [19, 20].

The peptide confidences provided by Proteome Discoverer 2.5 for the immunopeptidome samples were translated into numeric values ranging from 2 for “high” confident peptides, to 0.5 for peptides identified by “match between run” and the averages of the technical replicates were calculated for each peptide. Subsequently, the sum of these values per peptide were calculated for the biological replicates creating the confidence score. Frequently identified peptides are peptides with a confidence score larger than 4.2 (70% of the maximum possible confidence score). This cutoff value guarantees that frequently identified peptides have to be identified in all three biological replicates. Furthermore, only peptides detected in both technical replicates underwent further evaluation. The binding ranks for each peptide were calculated using the MixMHCpred tool (version 2.1) [21, 22]. Peptides were defined as HLA-specific binders when they are between 8-14 amino acids in length and have a binding rank of less than or equal to 2%. Venn diagrams of frequently identified and HLA-specific peptides were visualized with the R package ggvenn (version 0.1.9; Supplementary Figure 4d) or VennDiagram (version 1.7.3; Figure 4a). The protein abundance ratios (log2) of the proteome and the immunopeptidome datasets were plotted against each other and proteins significantly changed in both datasets (log2 abundance ratio greater than or equal to 1 and adjusted p-value less than or equal to 0.5) were colored and labeled with their gene symbol. Immune peptides with a log2 abundance ratio greater than or equal to 1 were colored red, while immune

peptides with a log2 abundance ratio less than or equal to 1 were colored blue. Peptides with unaffected MHC class I presentation but significant regulation in the proteome dataset were indicated as black dots.

Protein extraction and BCA assay

For Western blot analysis, cells, lung tissue or lung slices were lysed in RIPA buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodiumdeoxycholate, 0.1% SDS, supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche) for 20 min on ice. Total proteins were recovered upon centrifugation at 13 000 rpm for 20 min at 4°C. Protein concentration was determined by BCA assay using the BCA detection reagent according to manufacturer's instructions (Thermo Fisher Scientific) and an albumin standard (Thermo Fisher Scientific).

Western Blot analysis

20 µg protein of RIPA lysates were mixed with 6x Laemmli buffer (1 M Tris HCl, pH 6.8, 15% glycerol, 6% SDS, 1% bromophenol blue). Samples were separated on 10-15% SDS-PAGE gels and run at 90-120 V for 60-90 min. Gels were blotted onto polyvinylidene difluoride membranes at 250 mA for 90 min on ice. Membranes were blocked in Roti®-Block (Carl Roth) for 1 h at room temperature and incubated with primary antibodies overnight at 4°C (see Supplementary Table S4 for antibody details). Upon washing of the blots for 5 min with PBST (PBS, 0.1% Tween-20) three times, blots were incubated with HRP-coupled secondary antibodies at a dilution of 1:20,000 for 1-2 h at room temperature and images were taken using the iBRIGHT FL1500 system (Thermo Fisher).

Supplementary Table S6: Antibodies for Western blot analysis

Antibodies	Source	Identifier
Rabbit polyclonal anti-LMP2	Abcam	ab3328
Rabbit polyclonal anti-LMP7	Abcam	ab3329
HRP-conjugated-anti-β-actin	Sigma-Aldrich	a3854
Anti-rabbit IgG HRP-linked	Cell Signaling Technology	#7074
Anti-mouse IgG HRP-linked	Cell Signaling Technology	#7076

RNA extraction and RTqPCR analysis

Total RNA from cells and mouse PCLS was isolated using Roti-Quick Kits (Carl Roth) and peqGOLD Total RNA Kits ((VWR International), respectively. The obtained RNA was reverse transcribed with annealing for 5 min at 25°C and elongation for 60 min at 37°C using random hexamers (Life Technologies). For NHBE cells, total RNA was isolated using the miRNeasy

Mini Kit according to the manufacturer's instructions (Qiagen). Concentrations were determined using a DeNovix® DS- 11 Spectrometer (DeNovix Inc). Total RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time RT-PCR was performed using a SYBR Green LC480 system (Roche Diagnostics). Target gene expression was normalized to the mean of the two housekeeping genes *RPL19, RPL32* and *HPRT* using the delta ct method. All primer sequences are listed in Supplementary Table S5.

Supplementary Table S7: Primer sequences

Name	Acc. No.	Forward Primer (5'-3')	Reverse Primer (5'-3')
Mouse (SYBR Green)			
Psmb8	NM_010724.2	TGCTTATGCTACCCACAGAGACAA	TTCACTTTCACCCAACCGTC
Psmb9	NM_013585.2	GTACCGTGAGGACTTGTTAGCGC	GGCTGTCGAATTAGCATCCCT
Psmb10	NM_013640.3	GAAGACCGGTTCCAGCCAA	CACTCAGGATCCCTGCTGTGAT
Rpl19	NM_001159483.1	CGGGAATCCAAGAAGATTGA	TTCAGCTTGTGGATGTGCTC
Hprt	NM_013556.2	AATCCAGCAGGTCAGCAAAGAA	TGAAGGAGATGGGAGGCCA
Human (SYBR Green)			
PSMB8	NM_148919.4	AGTACTGGGAGCGCCTGCT	CCGACACTGAAATACGTTCTCCA
PSMB9	NM_002800.5	ATGCTGACTCGACAGCCTTT	GCAATAGCGTCTGTGGTGAA
PSMB10	NM_002801.4	TGCTGCGGACACTGAGCTC	GCTGTGGTTCCAGGCACAAA
IFNB1	NM_002176.4	AAGCAGCAATTTTCAGTGTCAG	CCTCAGGGATGTCAAAGTTCA
RPL32	NM_000994.4	CATCTCCTTCTCGGCATCAT	TAACCAATGTTGGGCATCAA
RPL19	NM_000981.4	GAGACCAATGAAATCGCCAATG	GCGGATGATCAGCCCATCTT
HPRT	NM_000194.3	TGAAGGAGATGGGAGGCCA	AATCCAGCAGGTCAGCAAAGAA

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